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UNDERSTANDING FLORAL SCENT IN A COEVOLVED PLANT-POLLINATOR SYSTEM: THE INHERITANCE OF COMPLEX TRAIT VARIATION IN *LITHOPHRAGMA* HYBRIDS

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Table of Contents

Table of Figures

Abstract

Understanding Floral Scent in a Coevolved Plant-Pollinator System: The Inheritance of Complex Trait Variation in Lithophragma Hybrids

Mia Tayler Waters

Floral volatiles are often used by plants to attract the subset of floral visitors that are mostly likely to contribute to pollination. Consequently, the composition and complexity of floral scents differ greatly among plant species. Recent studies of woodland stars, *Lithophragma* (Saxifragaceae), have found that species in this genus often have scents composed of an unusually large number of compounds, and even populations of the same species can differ extremely in the composition of scents. Here, I assess how the composition and complexity of floral scents change when closely related species of woodland stars hybridize. I evaluated floral scent in experimentally produced hybrids between two sister taxa, *L. parviflorum* and *L. affine*. Most F_1 and F_2 hybrids produced scents that were intermediate mixtures of the two parental species, but the scents of hybrids were often novel in the relative proportions of compounds they produced. Moreover, some hybrids produced scents with either fewer or more compounds than found in any parental individual. Alteration of the mix and complexity of monoterpenes contributed greatly to the variation among hybrid individuals, but most individuals produced scents composed of compounds arising from at least several biochemical pathways. Hence, the

chemical profiles of these hybrids suggest the possibility of transgressive segregation of this complex floral trait. These results suggest that natural hybridization in woodland stars would produce novel scent combination that could alter the interactions with their coevolved *Greya* moth pollinators and other visiting insects. Because the distributions of many plant species are rapidly changing, altered interactions with pollinators through novel scent combinations in hybrids could become common in many species-rich taxa capable of hybridization.

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Introduction

Floral signals mediate communication and interactions with their pollinators and thus are often under strong selection (Schiestl and Johnson 2013; Runquist *et al.* 2016; Borghi *et al.* 2017; Schiestl *et al.* 2018). Plants have evolved a wide range of signals (Raguso 2004) involving floral morphology (e.g., Bradshaw *et al.* 1998; Frame and Durou 2001; Thompson *et al.* 2013, 2017; Jager and Peakall 2016), floral color (e.g., Lunau and Maier 1995; Lunau 1996; Gumbert 2000; Peterson *et al.* 2015) and floral scent (e.g., Dotterl *et al.* 2006; Wright and Schiestl 2009; Dormont *et al.* 2014; Martin *et al.* 2017). The more than 1700 volatile organic compounds that are emitted by flowers vary widely across genera in their combinations, proportions, and effects on the attraction of potential pollinators (Knudsen *et al.* 2006; Jhumur *et al.* 2007; Okamoto *et al.* 2015).

These compounds may attract a suite of floral visitors, thereby forming complex networks of interactions between plants and animals (e.g., Bascompte *et al.* 2003; Lewinsohn *et al.* 2006; Olesen *et al.* 2006, 2007; Kantsa *et al.* 2018), or the compounds may be highly specialized as "private channels" of communication between a plant species and its obligate pollinator (e.g., Raguso 2008; Chen *et al.* 2009; Svensson *et al.* 2010; Soler *et al.* 2010). Floral scent is often used by floral visitors as an honest signal of potential nectar or pollen rewards (e.g., Irwin *et al.* 2004; Wright and Schiestl 2009; Balao *et al.* 2011; Haber *et al.* 2017), but is

sometimes used deceptively by plants to lure animals toward them dishonestly (e.g., Schiestl *et al.* 1999, 2003; Peakall *et al.* 2010; Xu *et al.* 2012; Peakall and Whitehead 2014). Furthermore, certain floral scent bouquets have been shown to repel facultative floral visitors in favor of obligate visitors (Junker and Blüthgen 2010) and to be simultaneously attractive to pollinators while repelling herbivores (Kessler *et al.* 2013). Floral scent can also be shaped by selection imposed by enemies such as seed predators and florivores/herbivores (Galen 1983; Kessler and Baldwin 2001; Bruce *et al.* 2005; Theis and Adler 2012) and potentially also through interactions with bacterial and fungal pathogens and mycophagous insects (Tabata *et al.* 2011; Huang *et al.* 2011). It is, then, not surprising that flowers have evolved such a wide range of volatile signals.

Floral scent may differ not only among species but also among geographically separated populations within species (Whitehead and Peakall 2009; Soler *et al.* 2011; Friberg *et al.* 2013, 2014; Bischoff *et al.* 2015). Local adaptation in species interactions arises when populations diverge geographically through differences in selection driven by inter- and intraspecific interactions, divergent physical environments, gene flow, genetic drift, and other genomic processes such hybridization or polyploidization, favoring different traits in different environments (Thompson 1994; Kawecki and Ebert 2004; Anderson and Johnson 2008; Leimu and Fischer 2008; Hereford 2009; Blanquart *et al.* 2013). The result can be a geographic mosaic of coadaptation among interacting species (Thompson 2013). How plants locally adapt to pollinators varies with the abundance and type of pollinators and their

frequency of visits (Schemske and Bradshaw 1999; Johnson and Steiner 2000; Kay and Schemske 2003; Thompson *et al.* 2010; Sun *et al.* 2016; Flores-Abreu *et al.* 2019) and likely depends on pollinator memory and the processing of stimuli (Chittka and Raine 2006) and geographic variation in the pollinators' optimal floral phenotype (Kay and Sargent 2009). Within the same plant species, there is therefore potential for local pollinator-mediated selection to favor different floral scent mixtures in different populations (Schiestl *et al.* 2011; Parachnowitsch *et al.* 2012; Byers *et al.* 2014; Nakahira *et al.* 2018).

Floral scents provide a useful model for complex trait evolution because these volatiles often consist of mixtures of discrete chemical compounds produced through identifiable biochemical pathways catalyzed by enzymes (Dudareva and Pichersky 2000; Dudareva *et al.* 2000; Klahre *et al.* 2011; Byers *et al.* 2014; Pichersky and Raguso 2016; Amrad *et al.* 2016; Cai *et al.* 2016). These mixtures can be thought of as complex signals, because together the compounds elicit a response (in this case pollinator attraction) but might not do so, at least in the same way, on their own (Hebets and Papaj 2005; Vereecken *et al.* 2010; Larue *et al.* 2016).

Hybridization of plants with distinctly variable or divergent complex traits can thus be a useful tool for assessing both the genetics of these traits and the ecological and evolutionary implications of their variability (Minder *et al.* 2007; Baack and Rieseberg 2007). Because the composition of floral scents often affects the range of pollinators that visit a plant's flowers (Jhumur *et al.* 2007; Schiestl 2010; Okamoto *et al.* 2015; Gross *et al.* 2016), hybridization among chemically divergent plant

populations could result in novel scent bouquets that disrupt the signals used by normal pollinators and simultaneously enhance signals that attract other pollinators. In such cases, hybridization can be the cause of speciation and adaptive radiations (Schluter 2001; Baack and Rieseberg 2007; Kagawa and Takimoto 2018; Lamichhaney *et al.* 2018; Gallego-Tévar *et al.* 2019).

In some cases, hybridization between plant species has led to reproductive isolation by way of a shift to a novel pollinator due to significant phenotypic changes in floral scent (Vereecken *et al.* 2010; Marques *et al.* 2016; Gervasi and Schiestl 2017; Wester *et al.* 2019). Hybridization zones where the hybrids have variable or intermediate floral scent profiles compared to their parents can also cause a breakdown of specificity in co-evolved pollinators (Svensson *et al.* 2016) or a breakdown of clear species boundaries (Pisano *et al.* 2019). The scent bouquets of interspecific hybrids vary widely in how similar, intermediate, or transgressive they are in relation to the parental species (Bischoff *et al.* 2014; Svensson *et al.* 2016). If hybrid scent is different enough to no longer attract the coevolved pollinator, the hybrid plants could only persist in the population if pollination by other animals was reliable.

The coevolving interactions between plants and prodoxid moths have become one of the useful models for understanding the coevolution of communication between plants and pollinating insects. These interactions have diversified to include both monocotyledonous plants (yuccas) (e.g., Pellmyr *et al.* 1996; Althoff *et al.* 2012) and eudicotyledonous plants (Saxifragaceae) (e.g., Thompson and Cunningham 2002;

Thompson and Rich 2011; Thompson *et al.* 2013, 2017). Prodoxid moths pollinate the same flowers in which they lay their eggs, and each prodoxid moth species is highly specific to one or a few plant species within a plant genus (Thompson and Pellmyr 1992; Thompson and Cunningham 2002; Thompson *et al.* 2010). *Greya* moths specialize on *Lithophragma* (woodland stars, Saxifragaceae) and live their entire lives on the plants. Adults take nectar and mate on the flowers, oviposit into the plant, the larvae hatch and eat the seeds, drop down to the ground to burrow into the underground bulbils to spend summer through winter, then they pupate rolled up in the basal leaves the next spring (Thompson and Cunningham 2002; Thompson *et al.* 2013). In turn, some *Lithophragma* species pollinated by prodoxid moths have become highly specialized for this interaction, and past studies have shown that populations and species of woodland stars and *Greya* moths covary geographically in morphology to varying degrees (Thompson and Fernandez 2006; Thompson *et al.* 2013, 2017).

Lithophragma plants attract their local prodoxid pollinators in the genus *Greya* at least partially through complex floral scents (Friberg *et al.* 2013, 2014). Recent experimental work has shown that *Greya obscura* and *Greya politella* moths prefer the chemical scent produced by their local *Lithophragma* plant population over the scent from other *Lithophragma* species. The moths preferentially orient toward the floral scent of their host, and *Greya politella* females also preferentially oviposit into flowers with the scent of their local host (Friberg *et al.* 2014). Although *Lithophragma* floral scents vary greatly among species and populations, there is little

variation within populations (Friberg *et al.* 2013, 2014) even under differences in nutrient availability (Friberg *et al.* 2017). This combination of little scent variation within *Lithophragma* populations and much variation among populations suggests that floral scent is canalized genetically and phenotypically locally to maintain communication with these important pollinators.

These past results provide an opportunity to evaluate how this genetically determined complex trait is altered when chemically divergent populations form hybrids. I performed hybrid crosses between two chemically divergent sister species of *Lithophragma* to determine how the complex floral scents inherited from these parents are expressed in their hybrid F_1 and F_2 offspring. I asked whether the hybrid offspring produce floral scents similar to or different from either of their parents, in terms of the number of compounds, the specific compounds present, and the proportional contribution (or ratios) of compounds making up the whole scent profile of each individual.

I hypothesized that the outcomes of these crosses could result in hybrids with any of three extreme scent profiles, with many intermediates possible:

1) Complete dominance of one parental species, effectively as a Mendelian trait: F_1 hybrids have the same floral scent profiles as one of the two parents, including the same number of compounds and the proportional contribution of each compound to the total scent. F_1 and F_2 offspring would show discrete mixes in approximately Mendelian ratios. This pattern could suggest that a supergene governs this complex trait as occurs, for example, in the wing coloration patterns of some

butterfly species (e.g., Joron *et al.* 2006; Booker *et al.* 2015; Charlesworth 2016).

2) "Intermediate" profiles: Similar to complete additivity, F_1 hybrids have floral scent profiles that are intermediate between the two parents in the number of compounds and the proportional contribution of each compound to the total scent profile. "Intermediate" here could be expressed in various ways, including offspring that produce a blend of all the compounds produced by both parents, or that produce particular groups of compounds from each parental species. If this trait were additive, F_2 offspring would show wider continuous variation than the F_1 generation.

3) Novel profiles: Hybrids have novel combinations of compounds, novel compounds, higher or lower numbers of compounds, or total scent emissions different from that of either parent species. This outcome could indicate transgressive segregation, a highly common phenotypic result in plant hybrids (Rieseberg *et al.* 1995, 1999), or possibly positive or negative epistasis (Phillips 2008).

Methods

Study system

Lithophragma affine and *L. parviflorum* form a monophyletic clade and are regarded either as sister species or as geographically divergent populations of one species (Taylor 1965; Soltis *et al.* 1992; Kuzoff *et al.* 1999). *L. affine* occurs from the Coast Ranges of California to the western side of the Cascade Mountains in the Pacific Northwest. *L. parviflorum* ranges from the foothills of the Sierra Nevada range through California, Oregon, Washington, and Idaho to parts of the Rocky Mountains.

Early molecular work has indicated that *L. affine* is embedded within *L. parviflorum* (Kuzoff *et al.* 1999), and preliminary experiments have shown that the two taxa readily form fertile hybrids when hand-crossed.

For this study, a population of *L. parviflorum* from eastern Washington State (Turnbull National Wildlife Refuge, 47°24'11.1"N, 117°34'06.2"W) was crossed with a population of *L. affine* from southwestern Oregon (Applegate River, 42°06'23.6"N, 123°05'53.8"W and 42°01'54.5"N, 123°05'27.4"W). The *L. parviflorum* population produces a floral mix strongly dominated by monoterpenoid compounds, whereas the *L. affine* population produces a more balanced mix of monoterpenoids, benzenoid esters, nitrogenous aromatics, and other groups in smaller quantities (Friberg *et al.* 2013, 2019).

Plant propagation and handling

Seeds from each parental population were collected from naturally pollinated plants at the field sites. One capsule was collected per maternal plant and given a unique family number. Crossing experiments done in preparation for this study showed that the plants are obligate outcrossers, as holds for most populations of most species of *Lithophragma.* Hence, the seeds within a capsule were either full- or half-siblings depending on whether the ovules were fertilized by pollen from the same or different paternal plants. These seeds were propagated as the parental generation and crossed by hand pollination to produce subsequent hybrid generations.

For each generation of hand-pollinated hybrids, seeds were collected as floral capsules ripened, and each capsule was given a unique identifying number. The seeds were counted on a printed grid under a microscope using a tally device. Fifty seeds from each cross were placed in separate packets for weighing on a Sartorius CPA microbalance. Thereafter, ten seeds from each seed family were planted in 2-inch pots with PRO-MIX HP (high porosity) soil.

Seedlings were sprouted in incubators (Percival, Boone, IA, USA, 15° C day, 10° C night, fluorescent lights set for a 14L:10D photoperiod), transplanted to individual 2-inch pots, then moved to a growth chamber (Conviron E-15, Pembina, ND, USA, 15° C day, 10° C night, fluorescent and incandescent lights set for a 14L:10D photoperiod, humidity 70% RH) until maturity. The plants were then moved to a greenhouse equipped with a swamp cooler and overhead lamps for the rest of their growth until senescence. The greenhouse was kept at ~20°C under semi-humid conditions to mimic the plants' native climate. Plants were watered three times per week and fertilized once per week with Dyna-Gro liquid 7-9-5 fertilizer containing 7% nitrogen (NH_4 and NO_3), 9% phosphorous (P_2O_5) and 5% potassium (K_2O), beginning one week after planting and ending when the plants stopped producing photosynthetic pigments. At this point the plants were allowed to dry out and the bulbils (root propagation bodies) were harvested and stored for later clonal growth. All hand-pollinations and scent collections were done during mid-day with plants acclimated to the greenhouse environment.

Crossing design

The parental plants were grouped randomly into seven quartets. Each quartet had two pairs of *L. affine* (A) and *L. parviflorum* (P), that were reciprocally crossed in all possible interspecific combinations – e.g., (P_1 x A_1), (A_1 x P_1); (P_2 x A_2), (A_2 x P_2) (Fig. 1A). The first letter in each pair is the female, and the second is the male. Within each quartet, every plant received pollen from, and provided pollen for, both plants of the other species. Thus every plant had two flowers hand-pollinated using two different plants of the other species. When possible, the first and third flowers on the scape were used for the crosses. The second flower was removed for scent collection. When crosses failed to develop properly, they were repeated. Because these populations were known to be obligate outcrossers, plants were not self-pollinated as controls. Even so, anthers were removed before dehiscing to eliminate any self-fertilization within a flower.

The F_1 progeny of the crosses were named to reflect their specific cross type, e.g., A_1P_1, P_1A_1, A_1P_2, A_2P_1, P_2A_2, A_2P_2, P_1A_2, P_2A_1. The F_1 plants were then crossed to produce the F_2 plants (Fig. 1B). Each generation of crosses was completed using the same protocols and reciprocal design as for the F_1 generation. With this design, a complete multi-generational quartet would include four parent plants, 8 F_1 plants, and 16 F_2 plants. No quartet was perfectly complete because offspring were lost at all stages: unsuccessful crossing, unsuccessful sprouting, the plant grew but did not flower, or, in rare cases, the scent was collected but the sample failed to produce a usable chemical profile due to equipment malfunction. When possible, these gaps were filled by repeating crosses, plantings, or scent collections.

Scent chemistry analysis

Although floral scent had already been shown in previous studies to differ between the two parental species (Friberg *et al.* 2013, 2014, 2019), floral volatiles were collected from the specific maternal and paternal plants used within each quartet to determine the exact floral scent profiles for each parental lineage. The scents of these parental plants were then used to evaluate how floral scents changed during the subsequent two generations of hybridization.

For each generation, floral scents were measured using two methods: solid phase micro-extraction ("SPME": field sampler 100-mm polydimethylsiloxane; Supelco (Sigma-Aldrich) Bellefonte, PA, USA) and dynamic headspace collection ("DH"), exactly following the protocols described in Friberg *et al.* 2013. SPME samples were collected by placing a cut flower inside a 2mL borosilicate glass vial sealed with a nylon resin oven bag (Reynolds®, Richmond, VA, USA). The headspace inside the vial equilibrated for 30 minutes before the SPME fibers were placed inside and exposed to the headspace air for another 30 minutes. Dynamic headspace samples were collected in the laboratory by encasing living scapes of ~10 flowers with 8cm x 14cm oven bags (Reynolds®, Richmond, VA, USA) and pulling ambient air through the bags with vinyl tubing connecting Teflon tube scent traps (filled with 10 mg of a Tenax GR® 10 mg filter, later eluted with hexane) to a Cole-Parmer (Vernon Hills, IL, USA) 65-mm direct-reading flowmeter at a steady rate of 200mL air per minute with a laboratory vacuum nozzle. These two methods (SPME and DH) capture similar scent profiles, but each is slightly more sensitive to certain compounds

(Friberg *et al*. 2013, 2019). SPME has traditionally been the preferred method for field sample collections, whereas dynamic headspace is preferred in a laboratory setting because 1) the samples can be stored for longer periods of time in hexane and be analyzed in batches; 2) this method allows for a more quantitative assessment of total scent emission, due to standardization in every sample with a known toluene standard; and 3) it allows for simultaneous sampling of multiple plant individuals. SPME was used to collect a complete set of samples from the parent and F_1 generations (n= 29, 57 respectively), and dynamic headspace was used to collect the main set of samples from the F_2 generation (n=171). A subset of F_2 plants (n=38) were also selected for SPME collections in order to directly compare the two methods.

Floral scents from each generation were analyzed using gas chromatography-mass spectrometry on a Hewlett-Packard (HP) 5890 gas chromatograph connected to an HP 5971 mass spectrometer (electronic ionization). The GC was equipped with an EC WAX polar column (30 m long, 0.25 mm × 0.25 μm film thickness; Grace, Deerfield, IL, USA). Helium was used as the carrier gas at a constant velocity of 1 mL min-1. The samples were analyzed starting with a 3-minute holding period at 60°C, and then the oven temperature was increased by 10°C per minute for 20 minutes until it reached a maximum temperature of 260°C, at which it stayed for a 7-minute hold before the analysis ended.

I manually integrated the number of floral compounds and concentration of each compound in the resulting chromatograms using the MS manufacturer's software

(G1034 Version C.02.00; Hewlett-Packard 1989–1993). I identified compounds using MS library suggestions (NIST/Wiley) and a list of volatiles known from previous studies to be emitted by *Lithophragma* plants (Friberg et al. 2014). From the list of 58 identified compounds, I removed 31 compounds that occurred in fewer than ten individuals (Table S1). These compounds either were too rare for interpretation or were user errors. Twelve of these compounds were found with only one of the scent collection methods (SPME or DH).

Statistical analysis

Comparison of scent collection methods: Scent from 38 F_2 individuals (46 samples) was used to compare the two volatile sampling techniques (DH and SPME) in five ways. First, I compared the distribution of the number of compounds found using either method, then plotted the correlation between methods for each plant sampled. I conducted a paired t-test (matched pairs analysis) in JMP Pro (JMP®, Version 14.0.0. SAS Institute Inc., Cary, NC, 1989-2019) to compare the differences between the mean number of compounds found by each method. I then assessed whether certain compounds were found more often, or only, with one method than the other. Finally, I tested for differences between the methods in multidimensional space by calculating Bray-Curtis dissimilarities among the samples using the vegan package (Oksanen et al. 2019), in R version 3.4.3 (R Core Team, 2013). I calculated the differences in two ways: first using the proportional contribution of each compound, and then again using the presence/absence of each compound. I tested for the effect of

method, cross type, and quartet on the scent variation using the PERMANOVA function.

Parental species: I evaluated differences among the 15 *L. affine* and 14 *L. parviflorum* parental individuals by comparing the proportional contribution of each compound to each individual's scent profile using Bray-Curtis dissimilarities and the PERMANOVA function in the vegan package, R version 3.4.3. I compared the number and classes of compounds found in *L. affine* and *L. parviflorum* and evaluated how often each compound occurred in the sampled plants. I then compared the mean contribution of each compound to the scent composition of each species overall.

Hybrid generations: As with the parental generation, differences in the proportional contribution of each compound among all generations were assessed using Bray-Curtis dissimilarities and a PERMANOVA analysis in the vegan package in R. In a series of three-dimensional NMDS plots, I partitioned the variance to visualize the effect of cross type and quartet. I also evaluated how the generations differed in scent variation among individuals by comparing the number of compounds present, their percentage of occurrence, and their mean percent contribution to the scent of each hybrid generation compared to the parents.

I assessed variation at the individual level within a quartet family by directly comparing the number of compounds present and their relative proportions of each F_1 and F_2 hybrid to its full siblings, its reciprocal cross type, and its parent and, for the F_2 individuals, its grandparents. In addition, collection of scent from multiple F2 full-

sibling plants allowed me to investigate the variation within a cross type in this generation. In the vegan package, I calculated the Bray-Curtis dissimilarities among full siblings to investigate with a PERMANOVA and NMDS plot whether being full siblings or of the same cross type is a predictor of variation across different parental lineages, based on the proportions of compounds being inherited.

Results

Comparison of scent collection methods: The number of compounds detected by solid phase microextraction (SPME) and dynamic headspace (DH) did not differ significantly for individuals analyzed by both methods (paired t-test, p=0.667, df=37). The detected number of compounds was significantly correlated between the two methods (Fig. 2, r^2=0.481, p <.0001, n=38). The number of compounds found in F_2 individuals by each method was nearly identical (SPME: mean =21.46, 1SD=6.4, n=38; DH: mean=21.04, 1SD=8.25, n=38).

Differences between methods were mostly in rarely detected compounds. Seven compounds were found only with DH and eight only with SPME. These compounds occurred in less than 5% of the samples, except for carvone, which was identified in 12.8% of the SPME samples. Each method was also more sensitive to a subset of compounds, but the differences were not greater than 50%. SPME identified four compounds >20% more often than DH and eight compounds 10-20% more often. DH identified two compounds >20% more often and three compounds 10-20% more

often. Hence, neither method was consistently more sensitive. (Supplementary Table S2, Fig. S1).

The scent collection method affected the multivariate scent composition, measured either as presence/absence of each compound or their proportional amount in each sample, significantly (PERMANOVA Presence/absence: $F_{1,66}$=5.966, p=.0003; Proportions: $F_{1,66}$=5.470, p=.0017), but the collection method accounted for only 7.3% and 6.5% of the variation, respectively. Within the F_2 samples, neither the proportional composition of scent nor the presence/absence of compounds differed significantly as a function of the direction of the cross, or among quartets (PERMANOVA Direction: $F_{7,66}$ =1.452, p = .083; Quartet: $F_{1,66}$=1.476, p=.192). Hence, the detected differences were overwhelmingly due to the overall species identity of the individuals.

Parental species: *Lithophragma affine* (A) and *L. parviflorum* (P) parental plants differed significantly in their chemical composition and the proportional amount of each present compound (Fig. 3A, PERMANOVA $F_{1,26}$=32.213, p<.001). Species accounted for 55.3% of the multidimensional variation. *Lithophragma affine* was also more variable among individuals than *L. parviflorum.* As A or P individuals were placed into the seven different quartets arbitrarily, the quartets accounted for only 1.45% of the variation, with the remaining variation found among individuals.

The scent of *L. parviflorum* plants was dominated by monoterpenoids (52% of the compounds detected). Esters and benzenoid esters made up another 22%, with a

diverse group of other compounds composing the remainder of the floral bouquet. In contrast, *L. affine* plants produced a floral scent that was more balanced among major chemical groups. Monoterpenoids and benzenoid esters each composed 22% of the mix, and six other major chemical groups each composed 6-11%. *Lithophragma parviflorum* scent included one benzenoid aldehyde (isoamylisovalerate, 4% of scent composition) not found in *L. affine*, while *L. affine* contained two sesquiterpenoids (11%) not found in *L. parviflorum* (Fig 3B). In total, *L. affine* emitted 18 detectable compounds, whereas *L. parviflorum* emitted 23 compounds. The mean number of compounds found per *L. affine* individual was half that found in *L. parviflorum* (A: mean=6, 1SD=2.0, P: mean=12, 1SD=2.9*). Lithophragma affine* emitted between 3-9 compounds per plant, whereas *L. parviflorum* either emitted 8-10 or 13-16 compounds (Fig. 3C), indicating that there might be two scent types in this species. There was more variation among individuals in *L. affine* than in *L. parviflorum*, but this variation was less than the variation between the two species.

The monoterpenoids found in *L. parviflorum* were a diverse mix, but three monoterpenes—alpha-pinene, beta-myrcene, and limonene—were found in every individual. Some other monoterpenoids were found in the majority of individuals, but all other compounds were found in half or less of the plants. (Fig. 4A). When combined as a floral bouquet, alpha-pinene dominated the overall scent of every individual, composing on average a little more than half of the mixture (Fig. 4B).

Only a few compounds found in *L. parviflorum* also occurred in *L. affine,* but most compounds found in the *L. affine* population also occurred in *L. parviflorum*

(Fig. 4A). Linalool and 3-hexen-1-ol were found only in *L. affine* but contribute relatively little to the overall scent. In this species, methyl salicylate and 1,3,3-trimethyl-7-oxabicyclo(4.1.0)heptan-2,5-dione were the two most common compounds, found in over 80% of the plants. These compounds each contributed less than 20% to the bouquet of *L. affine*, whereas trans-ocimene (which occurred in about 70% of plants) was the most common and abundant compound, making up about 35% of the average scent produced by the species (Fig. 4B).

Hybrid generations: The parental species, their F_1 hybrids, and the F_2 hybrids differed significantly in their chemical composition and the proportional amount of each present compound (Fig. 5, PERMANOVA $F_{3, 244}=14.995$, $p < .001$). The F_1 hybrids had scents that were distributed between those found in *L. parviflorum* and *L. affine,* but most F_1 hybrids were much more similar to *L. parviflorum* (Fig. 5, S2) due to the inheritance of a suite of monoterpenoids that were found only or mostly in this species. Alpha-pinene is the most notable of these compounds, as it was by far the most dominant compound (accounting for almost 60% of the scent emitted) in the *L. parviflorum* parent scent profile and was then found to be similarly dominating in both the F_1 and F_2 scent profiles. In fact, some F_1 hybrids had scents very similar to *L. parviflorum*, but no F_1 hybrids had scents very similar to *L. affine.* The F_2 hybrids showed a much broader distribution of scents, ranging fully across the span from *L. parviflorum* to *L. affine* and also showed a wider range of intermediate scents, Hence, F_2 plants expressed more of the underlying genetic variation.

Although most of the variation in floral scent occurred among species and generations, the quartets differed in how that variation was distributed among individuals (Fig. 6). Depending on the individual *L. affine* and *L. parviflorum* plants placed into a quartet as parents, and despite their scent profiles being relatively similar to each other within a species, the hybrid outcomes varied widely in each quartet, in no describable or predictable pattern. At the extremes, the F_1 and F_2 offspring of quartet 1 all clustered near *L. parviflorum*, whereas the F_1 and F_2 offspring of quartet 2 were broadly distributed in floral scents between *L. parviflorum* and *L. affine.* Analysis of the effects of direction of each cross (e.g., AP vs. PA) did not significantly affect the pattern of floral scent in F1 (PERMANOVA $F_{1,55}$=0.618, p =.613) or F_2 plants (PERMANOVA $F_{7,163}$=0.317, p =.081). In the F_2 generation, among-sibling variability was especially high, with some full seed siblings (produced from the same sire and dam plants with a single hand-pollination) emitting nearly identical scent profiles, and others being as different from each other as they were from unrelated F_2 plants from different cross types or quartets. (Fig. S3, PERMANOVA $F_{60,81}$=1.181, p =.052). Overall, then, the distribution of floral scents found in the F_1 and F_2 generations were a composite of the distributions found among the quartets.

The distribution of the number of compounds found in the F_1 plants were approximately fitted to a normal distribution and in the F2 plants, the number of compounds found in all individuals were significantly normally distributed (Fig. 7A, Shapiro-Wilk W test F_1: p=0.403, n=57 F_2: p=0.041, n=171). The F_1 plants emitted

between 2-20 floral compounds in a single plant and the F_2 plants emitted 1-20 compounds. The mean number of compounds in each generation was roughly intermediate between the two parental means; 11.8 compounds (1SD=4.0) for the F_1 plants and 10.2 (1SD=4.2) for the F_2 plants. The proportional amount of different compound groups in each hybrid generation more closely resembled *L. parviflorum* (Fig. 7B) in that they were dominated by monoterpenoids. 44% of the 36 compounds identified in the F_1 plants were monoterpenoids, as were 46% of the 44 F_2 compounds. The next most represented chemical group in the F_1 plants were benzenoid esters contributing 18% to the total bouquet, and esters in the F_2 plants that contributed 14% to their bouquet. Both esters and benzenoid esters were more strongly represented in *L. affine* (Fig. 3B), so it seems that while the hybrids did pick up more monoterpenoids from *L. parviflorum*, they also contain a higher proportion of compounds that were more characteristic of *L. affine*.

Two new compounds were found in the F_1 plants that were not in either of the parents: hexyl acetate and 3,6,6-trimethylbicyclo(3.1.1)heptan-2-one (Figure 4C,D) and in F_2 plants (Fig. 4E,F). Hexyl acetate has been reported in other *Lithophragma affine* populations, other plants from the Turnbull *L. parviflorum* population, and other *Lithophragma* species (Friberg *et al.* 2019). The compound 3,6,6-trimethylbicyclo(3.1.1)heptan-2-one hasn't been found previously in *Lithophragma*. In both generations, hexyl acetate was found in only four to ten percent of plants and contributed less than 0.2 percent to the total floral scent. 3,6,6-trimethylbicyclo(3.1.1)heptan-2-one occurred more often; in 47% of the F_1 plants and

22% of the F_2 plants. However, despite being more common, it also contributed only 0.01% or less to the overall scent in both generations.

Three additional rare compounds accounting for less than one per cent of the mean floral scent of each generation were either found only in the F_2 generation or lost in that generation (Fig. 4F). One of these was the novel compound, 1,8-cineole, which was found in the F_2 DH samples but not in the SPME samples (Table S1). Because DH samples were not taken for the parental and F_1 generations, it is not known if these compounds also occurred in those two generations. Conversely, two compounds from the other generations were not found in the F_2 plants. Because these compounds are produced in small amounts (each contributing less than 1% to the bouquet), their absence in other generations could have been due to production below the limits of detection.

Discussion

The variation in scent found among hybrids and quartets provides two insights into how hybridization of chemically divergent plant populations affects the diversification of floral scents in descendent generations. First, how complex floral scents are distributed among biochemical pathways can readily be reshaped to produce novel combinations in hybrids between chemically divergent plant populations. For these two species of woodland stars, most of the volatile combinations fell between the extremes of combinations found in the parental species, but a few hybrid combinations were even more novel. Moreover, some

hybrids produced either fewer compounds than the least fragrant *L. affine* (as few as one compound) and more than the most complex *L. parviflorum* (up to 20 compounds). The greater volatile complexity in some hybrids relative to *L. parviflorum* may be explained by inheritance of an additional set of compounds (such as the sesquiterpenoids) from *L. affine*, but it is more difficult to explain why some individuals have so few compounds. Possibly, genes for one biosynthetic pathway could have an epistatic effect on genes affecting volatile production through other pathways (Phillips 2008).

The overall range of scent variation found in these two woodland star species and their hybrids falls within the range of variation in floral scents found in *Lithophragma* in general. The three major clades within this genus vary in the combinations and the number of compounds they emit, and within each of these clades, there are pronounced regional differences in scent composition both among species and among populations (Friberg *et al.* 2019). That study identified very similar differences as shown here between *L. affine* and *L. parviflorum*; the dominant compound in all the measured populations of *L. parviflorum* was alpha-pinene, whereas in *L. affine* it was beta-ocimene. Both compounds are monoterpenes.

The combination of floral scents in hybrids with either fewer or more compounds than found in either parent, together with at least one compound in the F_2 generation not found in either parent, suggests the possibility of transgressive segregation in this complex trait. In plant hybrids, transgressive segregation has been found in floral morphology and color (Rieseberg *et al.* 1995; Bouck *et al.* 2007), environmental

tolerance (Gallego-Tévar *et al.* 2018), and secondary compound production (Orians *et al.* 2000). In a few other taxa, it has also been found for floral scent. For example, *Ophrys* orchid hybrids were found to produce new compounds, novel blends of compounds, and a higher number of compounds than their parents (Vereecken *et al.* 2010). In this case, the dramatic shift in floral blend led to decreased visits of the original pollinator (male bees) and attracted a novel pollinator. Putative hybrids from a mixed population of *Mandevilla laxa* and *M. pentlandiana* showed transgressive segregation in floral scent, where their scent blends extended beyond that of the parental species (Pisano *et al.* 2019). Again, these are two species that have different scent compositions that attract their pollinators; nocturnal hawkmoths on *M. laxa* and diurnal Hymenoptera on *M. pentlandiana.* In hybrids between *Ipomopsis tenuituba and I. aggregata* (Bischoff *et al.* 2014), the production of a compound important for the attraction of a particular pollinator is altered. *Ipomopsis tenuituba is* distinct from its close relative *I. aggregata* because, in addition to the terpenoids that the two species share, it produces the compound indole which attracts its major hawkmoth pollinator (Bischoff *et al.* 2015). Hybrids emitted an amount of indole intermediate between the two parents, whereas the other compounds varied in both novel and intermediate ways.

Effects of hybridization in other chemically divergent Saxifragaceae have been studied, but for morphological traits rather than for floral scents. In *Asimitellaria* (Saxifragaceae), several species occur in sympatry with different floral scent profiles and different pollinating fungus gnats that are specialized to prefer the scent of one

species over the other (Okamoto *et al.* 2015). The scent in this genus acts as a strong pre-mating barrier to hybridization that has likely led to speciation multiple times in the phylogeny, but the species can produce hybrids if hand-pollinated (Okuyama and Akashi 2013). Floral scent changes in these hybrids have not yet been investigated, but the hybrids differ in the number of stigma lobes, and number of flowers per inflorescence were intermediate to that of the parents in the F_1 generation and spanned (and in several cases exceeded in either direction) the range of variation between the two parents (Okuyama and Akashi 2013). In an earlier study on *Saxifraga* (Saxifragaceae) hybrids, qualitative morphological traits (glands on short hairs and sepals, and celia on leaves and sepals) were either intermediate or overlapping with the parental species (Gugerli 1997).

The second insight from my study is that some groups of compounds can have a disproportionate effect on the mixture of volatiles in hybrids. Consistent with previous studies, *L. parviflorum* was dominated by monoterpenoids, whereas the *L. affine* bouquet was more balanced in the proportional contribution of its different compound groups (Friberg *et al.* 2013, 2016). Generally in *Lithophragma*, the PAR clade is dominated by monoterpenes, but in varying amounts among the species, and with different monoterpenes serving as the major compound in different species (Friberg *et al.* 2019), like alpha-pinene in *L. parviflorum*, beta-ocimene in *L. affine*, and linalool in *L. bolanderi* (CAM clade). These compounds, along with limonene, are some of the most widespread and abundant compounds found in angiosperms and insects (Schiestl 2010). Monoterpenoid compounds play an important role in the

floral scent composition of *Asimitellaria* as well (Okamoto *et al.* 2015), with 23 monoterpenes out of 27 total compounds found. Linalool and (Z)-linalool oxide were shared among all species, but the remaining 25 compounds were found only in certain species. Monoterpenoids are produced by two major pathways in plant cells (Chen 2003; Tholl *et al.* 2005; Dudareva and Pichersky 2006; Pichersky and Raguso 2016) but only one of them, the MEP pathway, produces volatile terpenes, and does so in a rhythm with the circadian clock, thus influencing the timing of terpene synthesis and emission for different purposes (Dudareva *et al.* 2005).

Plants have evolved tens of thousands of terpenes for specialized purposes, with more likely still unknown (Pichersky and Raguso 2016). Floral scent is often emitted in highest quantities from the petals, with a different set of compounds emitted by the green floral parts (Dudareva and Pichersky 2006; Dudareva *et al.* 2013); this has been shown in *Lithophragma* as well (Friberg *et al.* 2016). Many monoterpenes are significantly associated with herbivory, and might thus have evolved within that context, with the plants co-opting the language of herbivorous insects, or vice versa (Schiestl 2010). Now as floral attractants for pollination, they might have evolved to be "multifunctional compounds" (Galen *et al.* 2011; Raguso *et al.* 2015). The adoption of these defense compounds as an attractive signal has been documented for every obligate pollination mutualisms between plants and insects (Pichersky and Raguso 2016). Furthermore, certain terpenoid compounds have been shown to function both as an attractant to obligate pollinators while repelling facultative visitors (Junker and Blüthgen 2010). It's uncertain if this is the case in *Lithophragma*,

but terpenoids likely play a significant role in the recognition by *Greya* moths to their preferred local host plants (Friberg *et al.* 2014), since there is so much geographic and species variation in the amount, number, and type of terpenes found.

Overall, these results provide implications for the evolutionary outcome of natural hybridization events as species ranges shift (Weber *et al.* 2018), in that the *Lithophragma* hybrids might emit intermediate or more variable scent bouquets that their coevolved pollinators might not recognize. They could potentially even experience a phenotype shift drastic enough to attract new pollinators or totally cease the attraction of *Greya*, thus breaking down their mutualistic relationship. Specificity for floral cues has been documented to vary in other plants coevolved with seed parasitizing pollinators, such as in the *Yucca* - *Yucca* moth system. This is a classic coevolutionary system similar to that of *Lithophragma – Greya,* as it is another example of plants coevolved with obligate prodoxid moths. In *Yucca filamentosa*, variation in the floral scent composition among populations is not nearly as varied as in *Lithophragma*, nor does the variation found correspond with different pollinators or with geographic distance (Svensson *et al.* 2005). In hybrids among two sympatric Joshua Tree species, *Yucca brevifolia* and *Y. jaegeriana*, floral scents range intermediately between the two distinct parental species' scents, and in these hybrid zones, the pollinators do not seem to discriminate among these different blends, in the hybrids or among the two host species despite having higher oviposition success on their local host. This suggests that perhaps these moths are attracted by the suite of compounds that the two species share, and not by their differing compounds

(Svensson *et al.* 2016). In other systems, different floral scent blends are essential for the attraction of insects to their specific host species within a genus. This is true for *Epicephala* moths that are seed parasites and pollinators on *Glochidion* trees in Japan and Taiwan (Okamoto *et al.* 2007). Fig wasps also have shown high specificity for the volatile profiles of their host fig species, where each fig species attracts one particular wasp species (Hossaert-McKey *et al.* 1994; Song *et al.* 2001; Yokoyama 2003; Hossaert-McKey *et al.* 2010).

It is imaginable that if *Greya* moths are attracted to their local host plants' unique floral blend (Friberg *et al.* 2016), and if hybridization occurs, the interaction might break down if the hybrid phenotypes proliferated through a population (such as in Svensson *et al.* 2016; Pisano *et al.* 2019). This of course might not be the case at all- it depends on whether *Greya* are attracted by precise cocktails of scent compounds or solely by independent compounds of importance, with the rest being background noise (Riffell *et al.* 2014). The interaction might be able to persist if the moths re-learn or adapt to the novel local cue (Goyret *et al.* 2008; Schiestl and Schlüter 2009; Wright and Schiestl 2009).

At a time when many species are undergoing changes in their geographic ranges (Parmesan and Yohe 2003; Higgins *et al.* 2003; Van der Putten 2012; Cunze *et al.* 2013), hybrid plants that produce novel scents, and consequently attract different combinations of pollinators, have the potential to alter patterns of gene flow within and among plant species in many ecosystems worldwide.

Figures

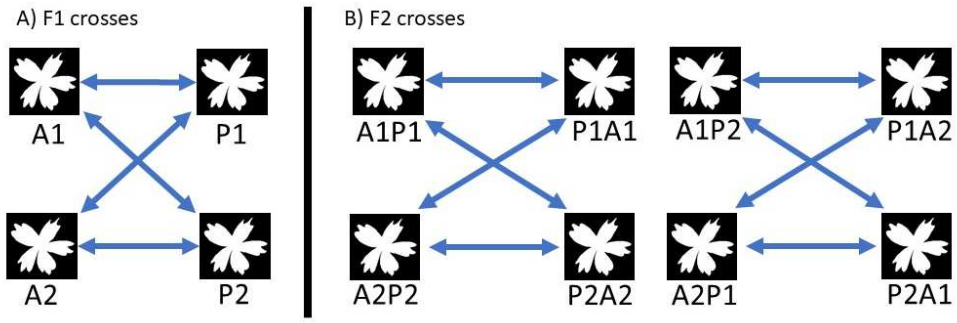

Figure 1. A) Diagram of crosses between parental plants. Two Lithophragma affine (A1 and A2) plants were reciprocally crossed with two Lithophragma parviflorum (P1 and P2) plants, producing eight F_1 plants. This process was replicated in seven quartets to maximize the number of different parental genotypes. **B)** Diagram of reciprocal crosses between F_1 plants to produce the F_2 generation. This process was replicated in the same seven quartets as the parental crosses to maintain lineages, with 16 crosses completed per quartet.

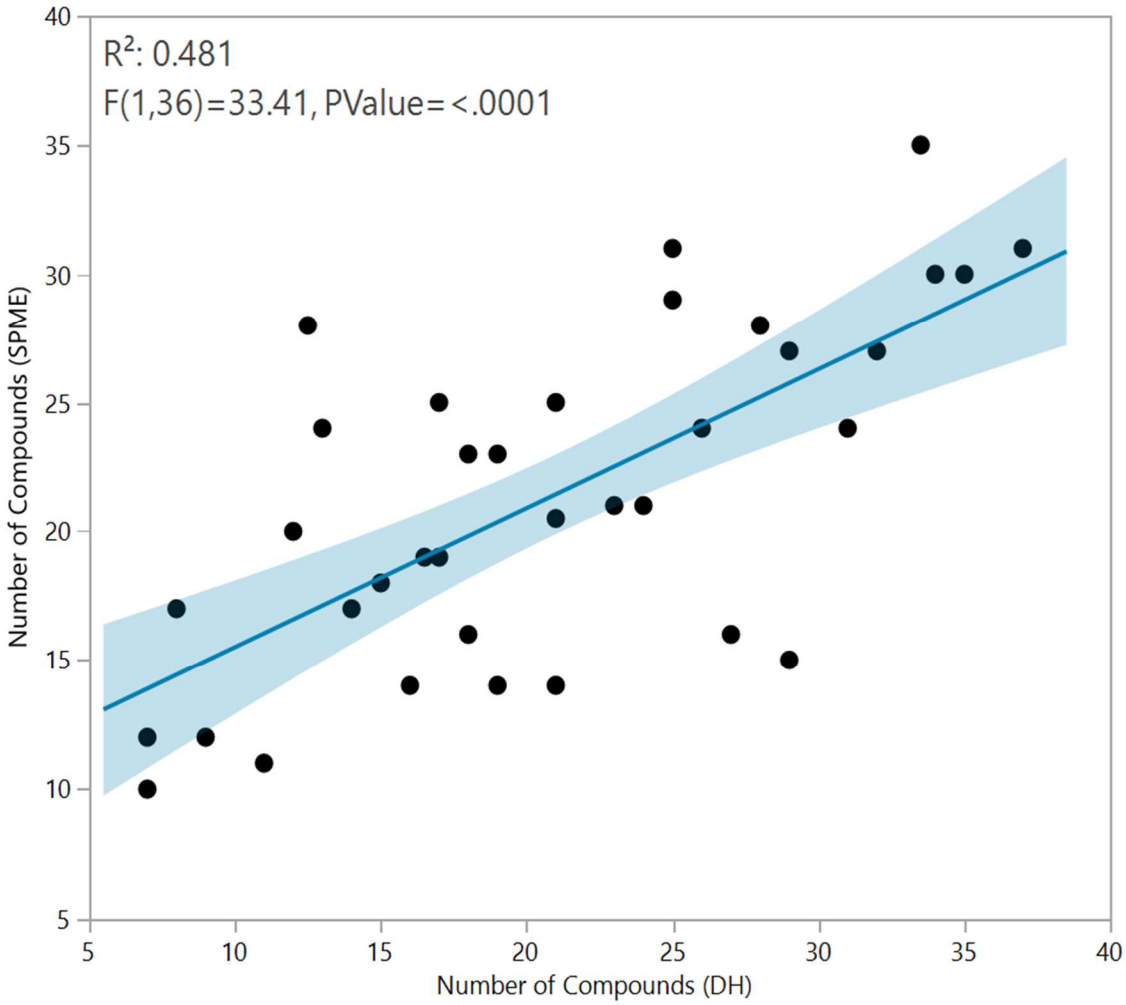

Figure 2. Pearson correlation of the number of compounds found using two scent collection methods*:* dynamic headspace (DH) and solid-phase microextraction (SPME). Each point represents one individual plant from which scent was collected using both methods within the same growth and flowering period. The ± 95% confidence limits around the regression line are shaded.

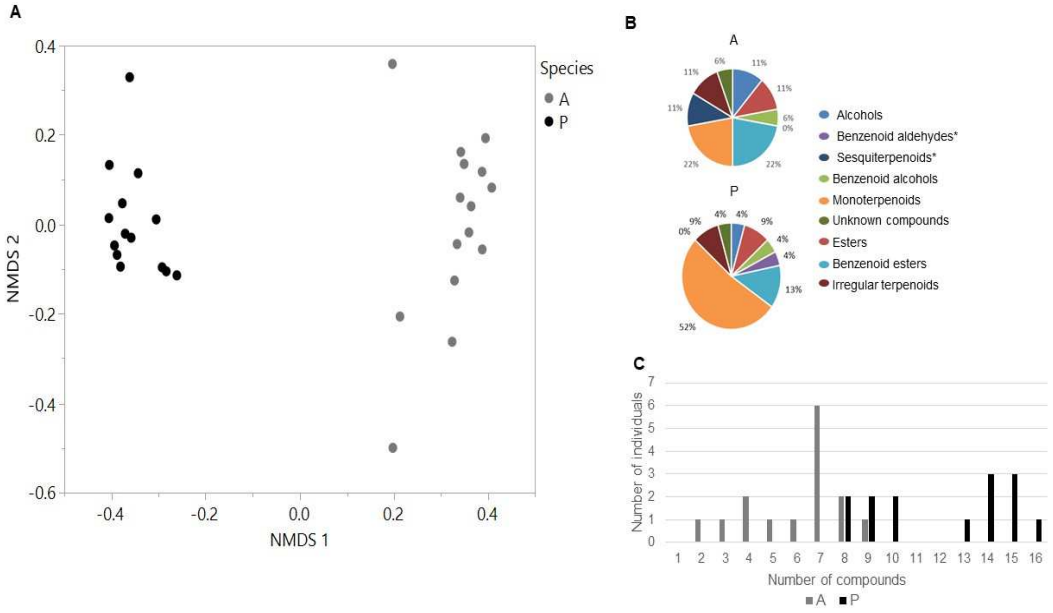

Figure 3. A) Non-metric multidimentional scaling (NMDS) plot of the *L. affine* (A) and *L. parviflorum* (P) parental plants grouped by Bray-Curtis dissimilarity for proportional contribution of each volatile compound to a plants' floral scent. Each point represents a plant. **B**) The proportions of compounds from each chemical group found in the *L. affine* (A) and *L. parviflorum* (P) parental plants all together, out of a total of 18 compounds found in A and 23 compounds found in P. Note that benzenoid aldehydes only occurred in *L. parviflorum*, and sesquiterpenoids only occurred in *L. affine*. **C**) Distribution showing the number of different compounds found per individual in the 15 *L. affine* (A) and 14 *L. parviflorum* (P) parental plants.

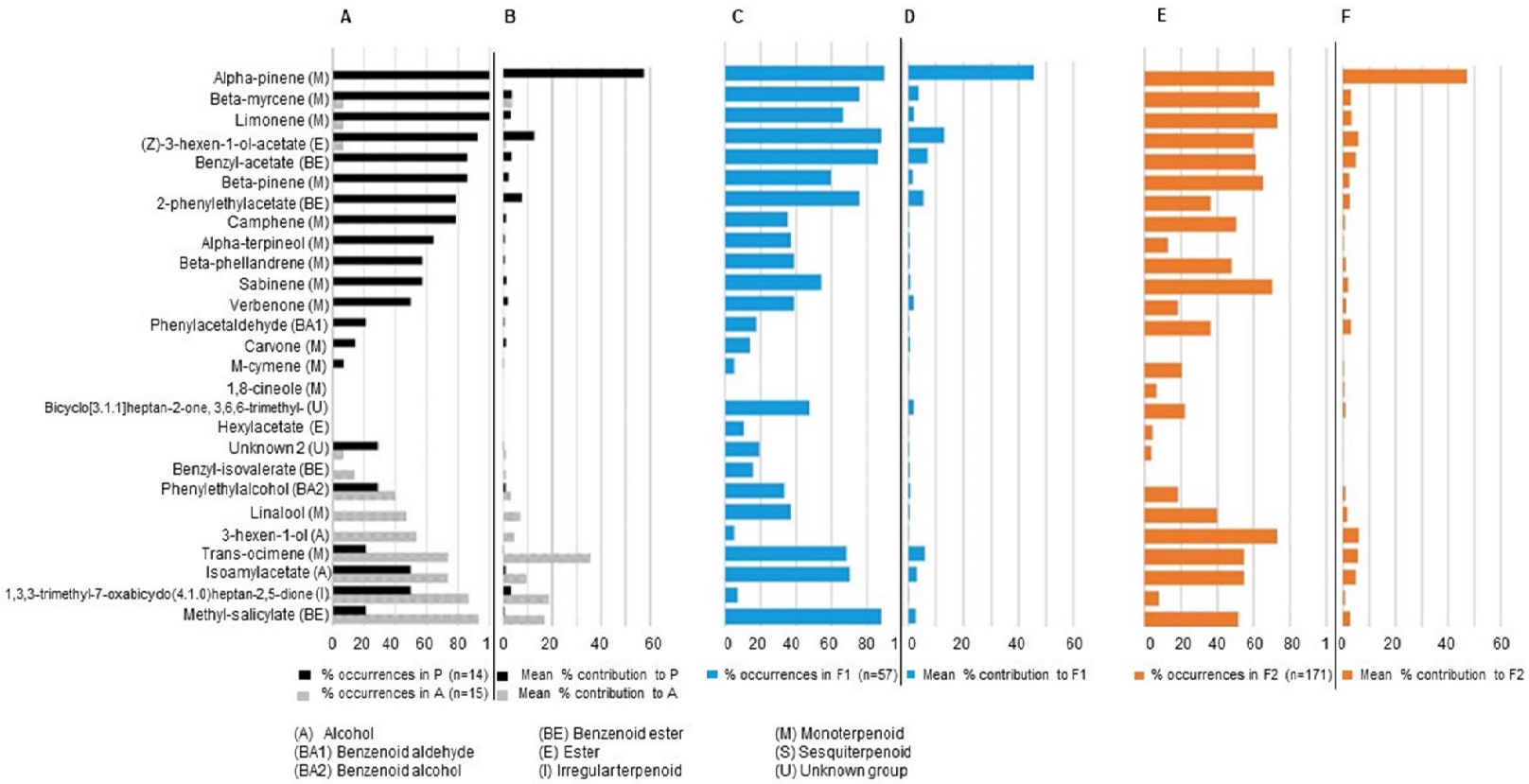

Figure 4. Constituents of the scent composition of the parental species *L. affine* (A, grey bars) and *L. parviflorum* (P, black bars), the F_1 hybrids (blue) and F_2 hybrids (orange). All compounds (excluding compounds found in fewer than 10 individuals) are included with the initial of their compound group in parentheses. Panels **A, C, and E** show the percentage of individuals in which each compound was found, and **B, D, F** show the mean percentage contribution of each compound to the total scent bouquet in the P, F_1, and F_2 generations, respectively.

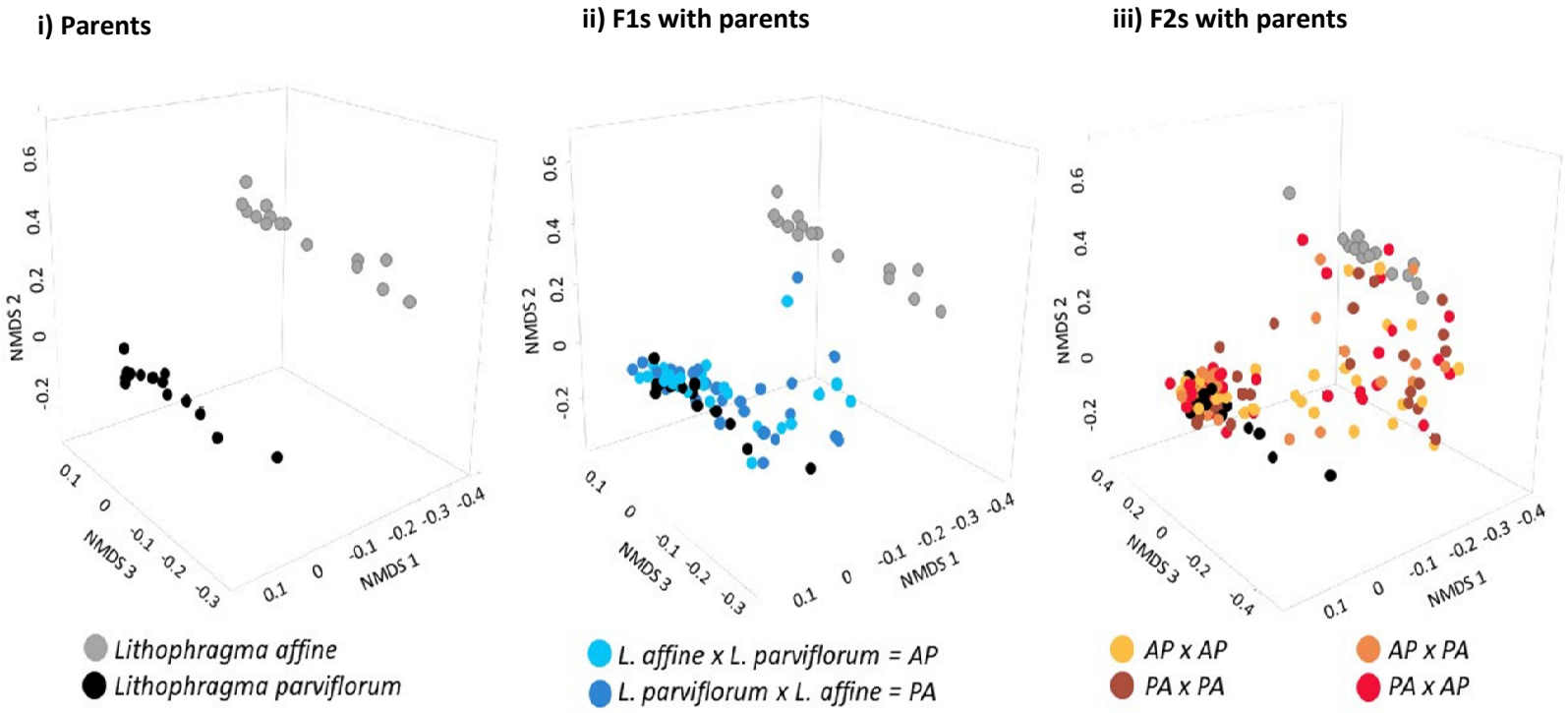

Figure 5. Non-metric multidimensional scaling (NMDS) plot showing the pattern of clustering (by Bray-Curtis dissimilarity in relative proportions of compounds calculated among all individuals) of floral scents for plants in the parental **(i)**, F1 **(ii)**, and F2 **(iii)** generations: L. affine (grey), L. parviflorum (black), F1 hybrids (blue hues: partitioned into AP (light blue) and PA (dark blue)), and F2 hybrids (orange hues: partitioned into four colors: APxAP (yellow), APxPA (orange), PAxAP (red), PAxPA (brown)). Note that the axis for NMDS 3 in the third panel is slightly longer than the first two- this is because the variation of the F2s extended farther in this dimension than the other two generations.

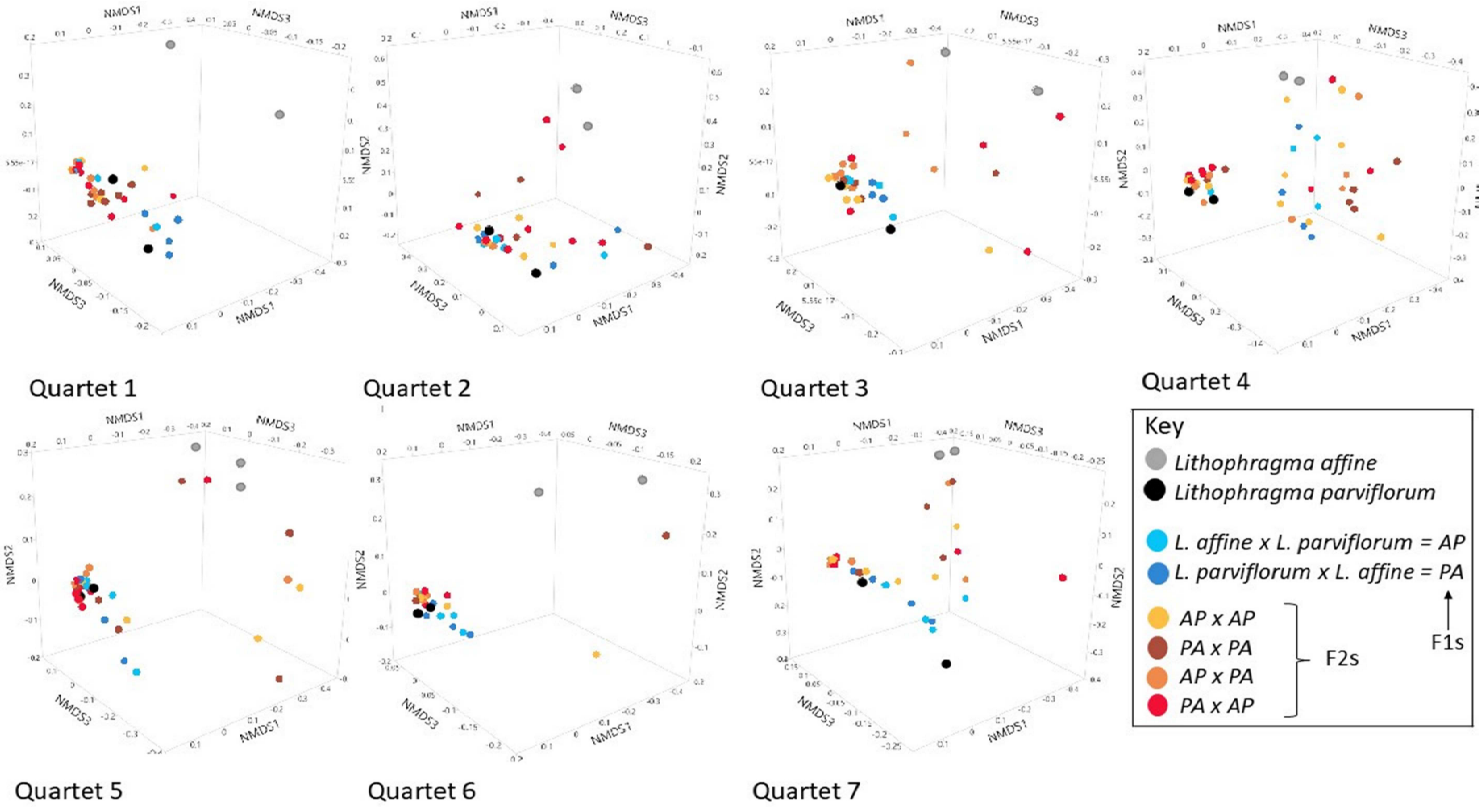

Figure 6. Non-metric multidimensional scaling (NMDS) plot showing the pattern of clustering (by Bray-Curtis dissimilarity in relative proportions of compounds calculated among all individuals) of floral scents for all plants, further partitioned to highlight the differences among the seven arbitrarily established quartets. Figure incorporates the same color coding and three-dimensional orientation as in Figure 5. Each quartet includes two parents of each species, and a minimum of 8 F1s and 16 F2s (in most of these quartets scent was collected from multiple seed siblings, thus the numbers could be higher).

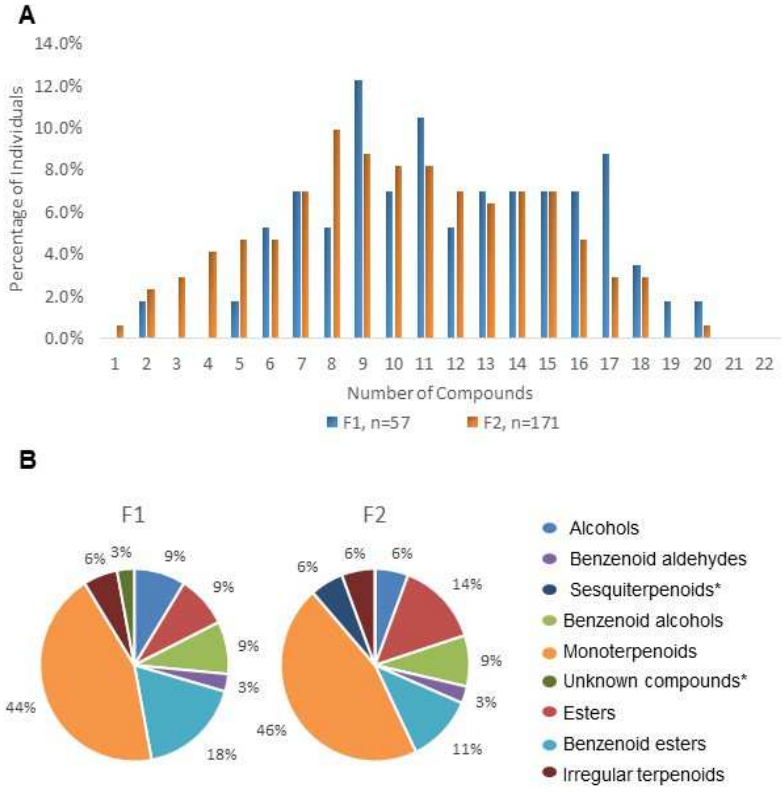

Figure 7. A) Distribution showing the number of different compounds found in the F1 and F2 hybrid plants. In the F1 plants (blue, n=57 individuals), the mean number of compounds was 11.8 with a standard deviation of 4.0. The F2 plants (orange, n= 171 individuals) had a similar mean of 10.2 and standard deviation of 4.2. **B)** The proportion of the overall scent of each hybrid generation composed from each of the compound groups found in the parents. Note that sesquiterpenoid compounds were only identified in the F2 generation and several unknown compounds were identified only in the F1 generation.

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